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Production of L-alanyl-L-glutamine by recycling *E. coli* expressing α-amino acid ester acyltransferase

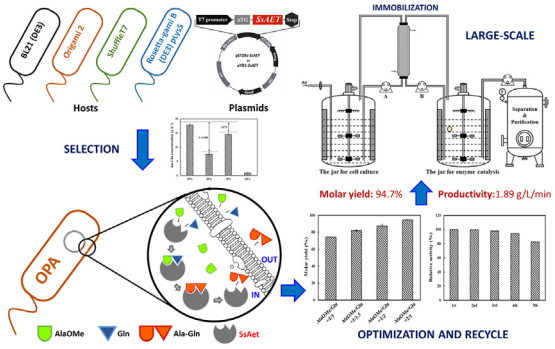
Yimin Li [a], Wenjie Yuan [a,*], Jiaoqi Gao [a], Chao Fan [b], Wenzhong Wu [b], Fengwu Bai [c]

[a] School of Life Science and Biotechnology, Dalian University of Technology, Dalian 116024, China
[b] Dalian Innobio Biological Co., LTD, Dalian 116600, China
[c] School of Life Science and Biotechnology, Shanghai Jiaotong University, Shanghai 200240, China

HIGHLIGHTS

- Optimal recombinant strain OPA expressed SaAet with high enzyme activity.
- The Ala-Gln productivity by OPA achieved 1.89 g/L/min.
- OPA maintained high yields and enzyme stabilities after several cell recycling.
- Recycling OPA is the cost-efficient and environmentally friendly approach.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, the optimum induction and reaction conditions, and fermentation process of producing Ala-Gln by *E. coli* Origami 2 overexpressing α-amino acid ester acyltransferase (OPA) were investigated. Besides, the Ala-Gln synthesis by OPA achieved the maximum molar yield of 94.7% and productivity of 1.89 g/L/min due to the extremely high enzyme activity. On this basis, repeated-cycle batch fermentation to produce Ala-Gln indicated that OPA could maintain high Ala-Gln yields and enzyme stabilities after several cell recycling. Consequently, the cost-efficient and environmentally friendly approach for Ala-Gln production by recycling OPA makes a great contribution to further industrial-scale applications.

1. Introduction

L-Glutamine (Gln), the conditionally essential amino acid, is the most abundant free amino acid of the plasma (the concentration is about 500–900 μM) and plays an important role in physiological functions for human beings (Brosnan, 2003; Wischmeyer et al., 2014), including nitrogen and carbon metabolism (Wernerman, 2004; Yuneva et al., 2007), protein and lipid synthesis (Corbet and Feron, 2015; Gouw et al., 2016), intestinal mucosa integrity

Abbreviations: Ala-Gln, L-alanyl-L-glutamine; Gln, L-glutamine; AlaOMe, L-alanine methyl ester hydrochloride; SsAet, α-amino acid ester acyltransferase from *Sphingobacterium siyangensis*; *SsAET*, the gene of SsAet; OPA, Origami 2-pET29a(+)-*SsAET*; BPA, BL21 (DE3)-pET29a(+)-*SsAET*; SPA, ShuffleT7-pET29a(+)-*SsAET*; RPA, Rosetta-gami B (DE3) pLysS-pYD1-*SsAET*; Lal, L-amino acid α-ligase; IPTG, isopropyl β-D-1-thiogalactopyranoside; PITC, phenylisothiocyanate.

* Corresponding author.
E-mail address: ywj@dlut.edu.cn (W. Yuan).

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maintaining (Yamamoto et al., 2017), and the like. However, Gln is rarely utilized to supply parenteral nutrition clinically due to its poor thermostability (heat-labile) and low solubility (36 g/L) (Fürst, 2001; Lacey and Wilmore, 1990), and the toxic 5-oxoproline (pyroglutamic acid) may be also generated from Gln by autoclaving sterilization. In order to solve these problems, Gln-containing dipeptides are supposed to be a reasonable choice (Hirao et al., 2013). L-Alanyl-L-Glutamine (Ala-Gln) has been clinically chosen as the supplement of Gln because of its high solubility (568 g/L), high thermostability, and high decomposition rate (Fürst, 2001, Fürst et al., 1997; Stehle et al., 1984).

Currently, chemical methods have been performed to produce Ala-Gln on an industrial scale. However, these chemical processes are somehow regarded to be complicated and less environmentally friendly in terms of the introducing/removing of some protecting groups for the target amino acid and complex manual manipulation (Akabori et al., 1961; Hou et al., 2008). Interestingly, a novel enzyme named L-amino acid α-ligase (Lal) was found in *Bacillus subtilis* by Tabata et al. (2005), which may generate Ala-Gln directly using unprotected L-amino acids as substrates under an ATP-dependent reaction. Compared with the traditional chemical synthesis approach, the Lal reaction is simpler and more environmentally friendly (Hashimoto and Tabata, 2009). However, the intracellular overexpression of Lal has a negative impact on the cell growth (Tabata and Hashimoto, 2007; Yagasaki and Hashimoto, 2008), and the yields of Ala-Gln by Lal are too low to be applied in the large-scale production.

Recently, Abe et al. (2011) reported an α-amino acid ester acyltransferase from *S. siyangensis* (SsAet), which is able to rapidly catalyze L-alanine methyl ester hydrochloride (AlaOMe) and Gln to synthesize Ala-Gln. Furthermore, different from Lal, SsAet can produce various dipeptides in the periplasm utilizing amino acids and their corresponding amino acid esters (acyl donors), which may reduce dipeptide degradation by proteases or peptidases (Arkowitz and Bassilana, 1994), and the accumulation of Ala-Gln is much higher than that by Lal. Consequently, the production of Ala-Gln from the novel SsAet is supposed to be a promising technical route in the future due to its high enzyme activity, few by-products and ATP-independence.

In spite of the outstanding advantages in the synthesis of Ala-Gln by recombinant *E. coli* with SsAet from *S. siyangensis* AJ2458 (Abe et al., 2011; Hirao et al., 2013), the following drawbacks cannot be ignored: (1) Inclusion bodies have an effect on the enzyme activity during the process of SsAet overexpression in recombinant *E. coli*; (2) Coupled with the slow catalytic reaction rate, the molar yields and production efficiency of Ala-Gln are still low. (3) Cell disruption, enzyme separation and purification, as well as the unrecyclable enzyme all increase total costs. Consequently, the whole recombinant cells with enhanced soluble SsAet protein represent a great promise for the efficient production of Ala-Gln at the current stage.

In this study, different hosts that help protein fold properly were utilized to express SsAet from *S. siyangensis* SY1 and produce Ala-Gln. Based on both ratios of soluble SsAet protein and enzyme activities, the Origami 2 strain overexpressing SsAet was selected for the further optimization of the culture and reaction conditions. Furthermore, the efficient production of Ala-Gln by recycling the recombinant strains lay a good foundation for its industrialization in the future.

2. Materials and methods

2.1. Strains and media

Strains were used in this study were shown in Table 1. Cultures were maintained on Luria–Bertani (LB) medium (0.5% yeast extract, 1% tryptone, and 1% sodium chloride) agar slants at 4 °C for routine use. For long-term preservation, cultures were stored at −80 °C in 20% glycerol. The liquid LB medium were used for strains growth and enzyme production in this study. For screening *E. coli* transformants, the media was supplemented with corresponding antibiotics (either 100 μg/mL ampicillin or 50 μg/mL kanamycin and the like).

2.2. Plasmid construction

The gene of SsAet (*SsAET*) was PCR-amplified using the *S. siyangensis* SY1 as a template with primers *SsAET-F* (5′-CGCGGATCCATGAAAAATACAATTTCGTGCC-3′), and *SsAET*-R (5′-CCGCTCGAGATCTTTGAGGACAGAAAATTCG-3′). After digestion by restriction enzymes (BamH I/Xho I), the PCR products were assembled into pET-29a(+) or pYD1 under T7 promoter. The expression plasmids, stored in *E. coli* DH5α, were transformed into the different *E. coli* hosts, BL21 (DE3), ShuffleT7, Origami 2 and Rosetta-gami B (DE3) pLysS, named as BPA, SPA, OPA and RPA, respectively. The recombinant strains were confirmed by enzyme digestion and sequencing.

2.3. Inducible expression of SsAet protein

The four recombinant strains were inoculated into LB medium containing corresponding antibiotics at 37 °C, 200 rpm. Enzyme

Table 1
Descriptions of strains and plasmids used in this study.

Strain or plasmid	Description	Source
Strains		
S. siyangensis SY1	Template of alpha-amino acid ester acyl transferase gene	CGMCC (1.6855)
DH5α	Genetic manipulation	Solarbio (C1100)
BL21 (DE3)	The most widely used host background for protein expression	Novagen (69450)
ShuffleT7	Enhancing capacity to correctly fold proteins with multiple disulfide bonds; streptomycin resistant	New England BioLab (C3026J)
Origami 2	Greatly facilitating cytoplasmic disulfide bond formation; tetracycline and streptomycin resistant	Novagen (71344)
Rosetta-gamiB (DE3) pLysS	Greatly facilitating the expression of genes that encode rare *E. coli* codons and cytoplasmic disulfide bond formation; kanamycin, chloramphenicol and tetracycline resistant	Novagen (71137)
BPA	pET29a- *SsAET* plasmid in BL21 (DE3); kanamycin selected	This study
SPA	pET29a- *SsAET* plasmid in ShuffleT7; kanamycin selected	This study
OPA	pET29a- *SsAET* plasmid in Origami 2; kanamycin selected	This study
RPA	pYD1- *SsAET* plasmid in Rosetta-gami B (DE3) pLysS; ampicillin selected	This study
Plasmids		
pET29a(+)	Empty vector; kanamycin resistant	Novagen (69871)
pYD1	Empty vector; ampicillin resistant	Addgene (73447)
pET29a(+)-*SsAET*	*SsAET* under the T7 promoter	This study
pYD1- *SsAET*	*SsAET* under the T7 promoter	This study

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